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(54) Title: NOVEL ANTIANGIOGENIC PEPTIDES, POLYPEPTIDES ENCODING SAME AND METHODS FOR INHIBITING ANGIOGENESIS

(57) Abstract

Mammalian kringle (5) fragments and kringle (5) fusion proteins are disclosed as compounds for treating angiogenic diseases. Methods and compositions for inhibiting angiogenic diseases are also disclosed.

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5'-AGCGGCGACGACGACGACAAG-3' (Ek-Cut-5p, SEQ ID NO:31) and 5'-CTTGTCGTCGTCGTCGCCGCT-3' (Ek-Cut-3p, SEQ ID NO:32 coding for an Enterokinase cleavage site) in 40 µL of BRL ligase and ligase buffer. An enterokinase site - mature stromelysin (Ek-Stromelysin) PCR fragment was generated using 1 µL of this ligation as a template, primers SEQ ID NO:29 and kinased SEQ ID NO:31, Ultma DNA polymerase and buffer at 94°C, 2 mins.; then 10 cycles of 94°C, 1 min.; 44°C, 1 min.; 72°C, 2 mins., then 72° for 7 mins.. The Ek-Stromelysin PCR fragment was gel purified.

The T7-ubiquitin and Ek-stromelysin PCR fragments were ligated together in BRL ligase and ligase buffer. A T7-ubiquitin-Ek-stromelysin PCR fragment was then generated using the ligation as template and Ultma DNA polymerase and the primers SEQ ID NO:28 and SEQ ID NO:29 at 94°C, 2' then 25 cycles of 94°C, 30 sec.; 42°C, 1 min.; 72°C, 6 mins., then 72°C for 7 mins.

A PCR fragment was generated using the stromelysin-pET3b plasmid template with the primers SEQ ID NO:26 and SEQ ID NO:30 with KlenTaq (AB Peptides, St. Louis, MO) and *pfu* DNA polymerases at 94°C, 2' then 15 cycles of 94°C, 30 sec.; 42°C, 2 mins.; 68°C, 20 mins.. This PCR fragment was mixed with the T7-Ubiquitin-Ek-Stromelysin PCR fragment and transformed into BRL DH5α maximum efficiency competent cells. Correct clones were identified by isolation of plasmid DNA, transfection into BL21(DE3), and expression studied as described above.

A PCR fragment for Ubiquitin-Ek was generated from a correct T7-Ubiquitin-Ek-Stromelysin expression plasmid with the primers SEQ ID NO:24 and SEQ ID NO:32 and *pfu* DNA polymerase at 94°C, 2' then 20 cycles of 94°C, 30 sec.; 40°C, 1 min.; 72°C, 3 mins., 72°C, 7 mins.. The fragment was purified over a Pharmacia S-400 HR Spin column and ligated to the VBC1 cassette using the Rapid DNA Ligation kit. A PCR fragment was generated using the ligation as template and the primers SEQ ID NO:24 and 5'-TGAAGAGCAAAAAAAAGCCCG-3' (SEQ ID NO:33) and *pfu* DNA polymerase at 94°C, 2 mins. then 20 cycles of 94°C, 30 sec.; 40°C, 1 min.; 72°C, 2 mins., 72°C, 7 mins.. The PCR fragment was kinased and ligated to Upet-H prepared for blunt, phosphatased cloning. The ligation was transformed into competent cells and colonies were screened by PCR as above. Plasmid DNA was sequenced to identify correct clones of UpET-Ubi.

WHAT IS CLAIMED IS:

1. A compound having the formula

A-B-C-X-Y

(I)

or a pharmaceutically acceptable salt, ester or prodrug thereof, wherein

A is absent or a nitrogen protecting group;

Y is absent or a carboxylic acid protecting group;

B is absent or is from 1 to about 197 naturally-occurring amino acid residues corresponding to the sequence from about amino acid position 334 to amino acid position 530 of SEQ ID NO:1;

C is R^1-R^2-R^3-R^4 wherein

R^1 is lysyl;

R^2 is leucyl or arginyl;

R^3 is tyrosyl, 3-I-tyrosyl or phenylalanyl;

R^4 is aspartyl; and

X is absent or is from 1 to about 12 naturally-occurring amino acid residues corresponding to the sequence from amino acid position 535 to about amino acid position 546 of SEQ ID NO:1 and homologues and analogues thereof.

2. The compound of Claim 1 wherein B is present and A, C, X and Y are as defined therein.

3. The compound of Claim 2 wherein X is present and A, B, C and Y are as defined therein.

4. The compound of Claim 3 wherein A and Y are present and B, C and X are as defined therein.

5. The compound of Claim 3 wherein A and Y are as defined therein and B-C-X is selected from the group consisting of

(a) the sequence from amino acid positions 355-543 of SEQ ID NO:1;

(b) the sequence from amino acid positions 355-546 of SEQ ID NO:1;

(c) the sequence from amino acid positions 443-543 of SEQ ID NO:1;

(d) the sequence from amino acid positions 449-543 of SEQ ID NO:1;

(e) the sequence from amino acid positions 454-543 of SEQ ID NO:1;

(f) the sequence from amino acid positions 443-546 of SEQ ID NO:1;

(g) the sequence from amino acid positions 449-546 of SEQ ID NO:1;

(h) the sequence from amino acid positions 454-546 of SEQ ID NO:1;

(i) the sequence from amino acid positions 525-535 of SEQ ID NO:1;

(j) the sequence from amino acid positions 529-535 of SEQ ID NO:1; and

(k) the sequence from amino acid positions 530-535 of SEQ ID NO:1.

6. The compound of Claim 5 wherein A is N-Ac and Y is $-NH_2$.

7. The compound of Claim 1 wherein X is absent and A, B, C and Y are as defined therein.

8. The compound of Claim 7 wherein X, A and Y are as defined therein and B-C is the sequence from amino acid positions 529-534 of SEQ ID NO:1.

9. The compound of Claim 1 wherein B and X are absent and A, C and Y are as defined therein.

10. The compound of Claim 9 wherein C is the sequence from amino acid positions 531-534 of SEQ ID NO:1.

11. The compound of Claim 1 wherein said compound has a molecular weight of between 0.5 and 25,000 kilodaltons as determined by reducing polyacrylamide gel electrophoresis or mass spectrometry analysis and an amino acid sequence substantially similiar to the corresponding amino acid sequence of SEQ ID NO: 1.

12. The compound of Claim 1 having an endothelial cell migration inhibition ED_{50} of about 100 to about 500 pM.

13. The compound of Claim 1 having an endothelial cell proliferation inhibition ED_{50} of about 100 to about 500 pM.

14. A compound having the formula

$$A\text{-}B_1\text{-}C_1\text{-}X_1\text{-}Y \qquad \textbf{(II)}$$

or a pharmaceutically acceptable salt, ester or prodrug thereof wherein

A is absent or a nitrogen protecting group;

Y is absent or a carboxylic acid protecting group;

B_1 is absent or is from 1 to about 176 naturally-occurring amino acid residues corresponding to the sequence from about amino acid position 334 to amino acid position 513 of SEQ ID NO:1;

C_1 is the sequence from amino acid position 514 to amino acid position 523 of SEQ ID NO:1; and

X_1 is absent or is from 1 to about 10 naturally-occurring amino acid residues corresponding to the sequence from amino acid position 524 to amino acid position 533 of SEQ ID NO:1 and homologues and analogues thereof.

15. The compound of Claim 14 wherein B_1 and X_1 are absent and A, C_1 and Y are as defined therein.

16. The compound of Claim 8 or 10 or 15 wherein A is N-Ac and Y is $-NH_2$.

17. A kringle 5 peptide fragment which has substantial sequence homology to a plasminogen fragment selected from human, murine, bovine, Rhesus monkey and porcine plasminogen.

18. A kringle 5 peptide fragment or fusion protein wherein the kringle 5 peptide fragment or kringle 5 fusion protein has a substantial sequence homology to human plasminogen.

19. A method of treating a disease in a patient in need of antiangiogenesis therapy comprising adminstering to a human or animal a therapeutically effective amount of a mammalian kringle 5 peptide fragment or kringle 5 fusion protein.

20. The method of Claim 19 wherein said mammalian kringle 5 peptide fragment or kringle 5 fusion protein is selected from the group consisting of a human, murine, bovine, Rhesus monkey and porcine kringle 5 peptide fragment or fusion protein.

21. The method of Claim 20 wherein said kringle 5 peptide fragment or kringle 5 fusion protein is a human kringle 5 peptide fragment or kringle 5 fusion protein.

22. The method of Claim 19 wherein said mammalian kringle 5 peptide fragment or kringle 5 fusion protein is a compound having the formula

A-B-C-X-Y

(I)

or a pharmaceutically acceptable salt, ester or prodrug thereof, wherein

A is absent or a nitrogen protecting group;

Y is absent or a carboxylic acid protecting group;

B is absent or is from 1 to about 197 naturally-occurring amino acid residues corresponding to the sequence from about amino acid position 334 to amino acid position 530 of SEQ ID NO:1;

C is R^1-R^2-R^3-R^4 wherein

R^1 is lysyl;

R^2 is leucyl or arginyl;

R^3 is tyrosyl, 3-I-tyrosyl or phenylalanyl;

R^4 is aspartyl; and

X is absent or is from 1 to about 12 naturally-occurring amino acid residues corresponding to the sequence from amino acid position 535 to about amino acid position 546 of SEQ ID NO:1 and homologues or analogues thereof.

23. The method of Claim 22 wherein said mammalian kringle 5 fragment or kringle 5 fusion protein is said compound wherein A and Y are as defined therein and B-C-X is selected from the group consisting of

(a) the sequence from amino acid positions 355-543 of SEQ ID NO:1;
(b) the sequence from amino acid positions 355-546 of SEQ ID NO:1;
(c) the sequence from amino acid positions 443-543 of SEQ ID NO:1;
(d) the sequence from amino acid positions 449-543 of SEQ ID NO:1;
(e) the sequence from amino acid positions 454-543 of SEQ ID NO:1;
(f) the sequence from amino acid positions 443-546 of SEQ ID NO:1;
(g) the sequence from amino acid positions 449-546 of SEQ ID NO:1;
(h) the sequence from amino acid positions 454-546 of SEQ ID NO:1;
(i) the sequence from amino acid positions 525-535 of SEQ ID NO:1;
(j) the sequence from amino acid positions 529-535 of SEQ ID NO:1; and
(k) the sequence from amino acid positions 530-535 of SEQ ID NO:1.

24. The method of Claim 22 said compound is said mammalian kringle 5 fragment wherein X is absent, A and Y are as defined therein and B-C is the sequence from amino acid positions 529-534 of SEQ ID NO:1.

25. The method of Claim 22 wherein said compound is said mammalian kringle 5 fragment wherein X and B are absent, and A, C and Y are as defined therein.

26. The method of Claim 23 or 24 or 25 wherein A is N-Ac and Y is $-NH_2$.

27. The method of Claim 19 wherein said disease is selected from the group consisting of cancer, arthritis, macular degeneration and diabetic retinopathy.

28. The method of Claim 27 wherein said disease is cancer.

29. The method of Claim 28 wherein said disease is selected from primary and metastatic solid tumors, carcinomas, sarcomas, lymphomas, psoriasis and hemagiomas.

30. The method of Claim 19 wherein said mammalian kringle 5 peptide fragment or kringle 5 fusion protein is a compound having the formula

$$A\text{-}B_1\text{-}C_1\text{-}X_1\text{-}Y \qquad (II)$$

or a pharmaceutically acceptable salt, ester or prodrug thereof, wherein

A is absent or a nitrogen protecting group;

Y is absent or a carboxylic acid protecting group;

B_1 is absent or is from 1 to about 176 naturally-occurring amino acid residues corresponding to the sequence from about amino acid position 334 to amino acid position 513 of SEQ ID NO:1;

C_1 is the sequence from amino acid position 514 to amino acid position 523 of SEQ ID NO:1; and

X_1 is absent or is from 1 to about 10 naturally-occurring amino acid residues corresponding to the sequence from amino acid position 524 to amino acid position 533 of SEQ ID NO:1 and homologues or analogues thereof.

31. The method of Claim 30 wherein said compound is said mammalian kringle 5 peptide fragment wherein B_1 and X_1 are absent, A, C_1 and Y are as defined therein.

32. A composition comprising an isolated single- or double-stranded polynucleotide sequence that encodes a kringle 5 peptide fragment or kringle 5 fusion protein having angiogenesis inhibiting activity.

33. The composition of Claim 32 wherein said polynucleotide sequence is a DNA sequence.

34. The composition of Claim 33 wherein said DNA sequence encodes an amino acid sequence selected from the group consisting of

(a) the sequence from amino acid positions 443-543 of SEQ ID NO:1;

(b) the sequence from amino acid positions 449-543 of SEQ ID NO:1;

(c) the sequence from amino acid positions 454-543 of SEQ ID NO:1; and

(d) the sequence from amino acid positions 355-543 of SEQ ID NO:1.

35. The composition of Claim 33 wherein said polynucleotide sequence encodes an amino acid sequence selected from the group consisting of SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, and SEQ ID NO:37.

36. A composition comprising a kringle 5 peptide fragment or kringle 5 fusion protein and a pharmaceutically acceptable excipient.

37. A method comprising implanting into a human or non-human animal a cell containing a vector, wherein said vector contains a DNA sequence encoding a kringle 5 peptide fragment or kringle 5 fusion protein and wherein said vector is capable of expressing said kringle 5 peptide fragment or kringle 5 fusion protein when present in said cell.

38. A method of making a kringle 5 peptide fragment comprising the steps of:

(a) exposing a mammalian plasminogen to elastase at a ratio of about 1:100 to about 1:300 to form a mixture of said plasminogen and said elastase;

(b) incubating said mixture; and

(c) isolating said kringle 5 from said mixture.

39. An isolated single- or double-stranded polynucleotide which encodes a mammalian kringle 5 peptide fragment or kringle 5 fusion protein having angiogenesis inhibiting activity.

40. The polynucleotide of Claim 39 wherein said mammalian kringle 5 peptide fragment or kringle 5 fusion protein encoded by said polynucleotide is selected from the group consisting of human, Rhesus monkey, bovine, murine, and porcine kringle 5 peptide fragment or fusion protein.

41. The polynucleotide of Claim 40 wherein said mammalian kringle 5 peptide fragment or kringle 5 fusion protein is a human kringle 5 peptide fragment or kringle 5 fusion protein.

42. The polynucleotide of Claim 39 wherein said mammalian kringle 5 peptide fragment or kringle 5 fusion protein is a compound having the formula B-C-X or B_1-C_1-X_1 wherein

B is absent or is from 1 to about 197 naturally-occurring amino acid residues corresponding to the sequence from about amino acid position 334 to amino acid position 530 of SEQ ID NO:1;

C is R^1-R^2-R^3-R^4 wherein

R^1 is lysyl;

R^2 is leucyl or arginyl;

R^3 is tyrosyl or phenylalanyl; and

R^4 is aspartyl;

X is absent or is from 1 to about 12 naturally-occurring amino acid residues corresponding to the sequence from amino acid position 535 to about amino acid position 546 of SEQ ID NO:1;

B_1 is absent or is from 1 to about 176 naturally-occurring amion acid residues corresponding to the sequence from about amino acid position 334 to amino acid position 513 of SEQ ID NO:1;

C_1 is the sequence from amino acid position 514 to amino acid position 523 of SEQ ID NO:1; and

X_1 is absent or is from 1 to about 10 natually-occurring amino acid residues corresponding to the sequence from amino acid position 524 to amino acid position 533 of SEQ ID NO:1 and complements thereof.

43. The polynucleotide of Claim 42 wherein said mammalian kringle 5 peptide fragment or kringle 5 fusion protein is a compound wherein B is present and C and X are as defined therein.

44. The polynucleotide of Claim 42 wherein said a mammalian kringle 5 peptide fragment or kringle 5 fusion protein is a compound wherein X is present and B and C are as defined therein.

45. The polynucleotide of Claim 42 wherein said mammalian kringle 5 peptide fragment or kringle 5 fusion protein is a fragment wherein B and X are present and C is as defined therein.

46. The polynucleotide of Claim 39 wherein said mammalian kringle 5 peptide fragment or kringle 5 fusion protein is selected from the group consisting of

(a) the sequence from amino acid positions 355-543 of SEQ ID NO:1;

(b) the sequence from amino acid positions 355-546 of SEQ ID NO:1;

(c) the sequence from amino acid positions 443-543 of SEQ ID NO:1;

(d) the sequence from amino acid positions 449-543 of SEQ ID NO:1;

(e) the sequence from amino acid positions 454-543 of SEQ ID NO:1;

(f) the sequence from amino acid positions 443-546 of SEQ ID NO:1;

(g) the sequence from amino acid positions 449-546 of SEQ ID NO:1; and

(h) the sequence from amino acid positions 454-546 of SEQ ID NO:1.

47. The polynucleotide of Claim 42 wherein said mammalian kringle 5 peptide fragment or kringle 5 fusion protein is a fragment wherein X is absent and B and C are as defined therein.

48. The polynucleotide of Claim 39 which is a DNA molecule.

49. The polynucleotide of Claim 39 which is an RNA molecule.

50. A vector comprising a polynucleotide which encodes a mammalian kringle 5 peptide fragment or kringle 5 fusion protein having angiogenesis inhibiting activity.

51. The vector of Claim 50 which is an expression vector.

52. The vector of Claim 51 wherein said mammalian kringle 5 peptide fragment or kringle 5 fusion protein encoded by said polynucleotide is a compound having the formula B-C-X or B_1-C_1-X_1 wherein

B is absent or is from 1 to about 197 naturally-occurring amino acid residues corresponding to the sequence from about amino acid position 334 to amino acid position 530 of SEQ ID NO:1;

C is R^1-R^2-R^3-R^4 wherein

R^1 is lysyl;

R^2 is leucyl or arginyl;

R^3 is tyrosyl or phenylalanyl; and

R^4 is aspartyl;

X is absent or is from 1 to about 12 naturally-occurring amino acid residues corresponding to the sequence from amino acid position 535 to about amino acid position 546 of SEQ ID NO:1;

B_1 is absent or is from 1 to about 176 naturally-occurring amion acid residues corresponding to the sequence from about amino acid position 334 to amino acid position 513 of SEQ ID NO:1;

C_1 is the sequence from amino acid position 514 to amino acid position 523 of SEQ ID NO:1; and

X_1 is absent or is from 1 to about 10 natually-occurring amino acid residues corresponding to the sequence from amino acid position 524 to amino acid position 533 of SEQ ID nO:1 and complements thereof.
and complements thereof.

53. The vector of Claim 52 wherein said mammalian kringle 5 peptide fragment or kringle 5 fusion protein is a compound wherein B and X are present and C is as defined therein.

54. The vector of Claim 52 wherein said mammalian kringle 5 peptide fragment or kringle 5 fusion protein is a compound wherein X is absent and B and C are as defined therein.

55. The vector of Claim 52 wherein said mammalian kringle 5 peptide fragment or kringle 5 fusion protein is selected from the group consisting of

(a) the sequence from amino acid positions 355-543 of SEQ ID NO:1;
(b) the sequence from amino acid positions 355-546 of SEQ ID NO:1;
(c) the sequence from amino acid positions 443-543 of SEQ ID NO:1;
(d) the sequence from amino acid positions 449-543 of SEQ ID NO:1;
(e) the sequence from amino acid positions 454-543 of SEQ ID NO:1;
(f) the sequence from amino acid positions 443-546 of SEQ ID NO:1;
(g) the sequence from amino acid positions 449-546 of SEQ ID NO:1; and
(h) the sequence from amino acid positions 454-546 of SEQ ID NO:1.

56. The vector of Claim 52 selected from the group consisting of pHil-D8, pET32a, pGEX-4T-2, Up-ET, UpET-Ubi, and pCYB3.

57. The vector of Claim 52 further comprising a host cell transformed with said vector.

58. The vector of Claim 57 wherein said host cell is a eukaryotic cell.

59. The vector of Claim 58 wherein said eukaryotic cell is *Pichia pastoris*.

60. The vector of Claim 57 wherein said host cell is a prokaryotic cell which is *E. coli*.

61. A method for making a soluble kringle 5 peptide fragment or kringle 5 fusion protein comprising the steps of:

(a) isolating a polynucleotide which encodes said kringle 5 peptide fragment;

(b) cloning said polynucleotide into an expression vector;

(c) transforming said vector into a suitable host cell; and

(d) growing said host cell under conditions suitable for the expression of said soluble kringle 5 peptide fragment or kringle 5 fusion protein.

62. A compound selected from the group consisting of

(a) A-Pro-Arg-Lys-Leu-Tyr-Asp-3-I-Tyr-Y;

(b) A-Pro-Arg-Lys-Leu-3-I-Tyr-Asp-Tyr-Y;

(c) A-Pro-Glu-Lys-Arg-Tyr-Asp-Tyr-Y; and

(d) A-Gln-Asp-Trp-Ala-Ala-Gln-Glu-Pro-His-Arg-His-Ser-Ile-Phe-Thr-Pro-Glu-Thr-Pro-Glu-Thr-Asn-Pro-Arg-Ala-Gly-Leu-Glu-Lys-Asn-Tyr-Y.

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FIG. 1(a) (SEQ ID NO:1)

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GLU PRO LEU ASP ASP TYR VAL ASN THR GLN GLY ALA SER LEU PHE
1               5                  10                  15

SER VAL THR LYS LYS GLN LEU GLY ALA GLY SER ILE GLU GLU CYS
            20                  25                  30

ALA ALA LYS CYS GLU GLU ASP GLU GLU PHE THR CYS ARG ALA PHE
        35                  40                  45

GLN TYR HIS SER LYS GLU GLN GLN CYS VAL ILE MET ALA GLU ASN
    50                  55                  60

ARG LYS SER SER ILE ILE ILE ARG MET ARG ASP VAL VAL LEU PHE
65                  70                  75

GLU LYS LYS VAL TYR LEU SER GLU CYS LYS THR GLY ASN GLY LYS
            80                  85                  90

ASN TYR ARG GLY THR MET SER LYS THR LYS ASN GLY ILE THR CYS
        95                  100                 105

GLN LYS TRP SER SER THR SER PRO HIS ARG PRO ARG PHE SER PRO
    110                 115                 120

ALA THR HIS PRO SER GLU GLY LEU GLU GLU ASN TYR CYS ARG ASN
125                 130                 135

PRO ASP ASN ASP PRO GLN GLY PRO TRP CYS TYR THR THR ASP PRO
            140                 145                 150

GLU LYS ARG TYR ASP TYR CYS ASP ILE LEU GLU CYS GLU GLU GLU
        155                 160                 165

CYS MET HIS CYS SER GLY GLU ASN TYR ASP GLY LYS ILE SER LYS
    170                 175                 180

THR MET SER GLY LEU GLU CYS GLN ALA TRP ASP SER GLN SER PRO
185                 190                 195

HIS ALA HIS GLY TYR ILE PRO SER LYS PHE PRO ASN LYS ASN LEU
            200                 205                 210

LYS LYS ASN TYR CYS ARG ASN PRO ASP ARG GLU LEU ARG PRO TRP
        215                 220                 225

CYS PHE THR THR ASP PRO ASN LYS ARG TRP GLU LEU CYS ASP ILE
    230                 235                 240

PRO ARG CYS THR THR PRO PRO PRO SER SER GLY PRO THR TYR GLN
245                 250                 255

CYS LEU LYS GLY THR GLY GLU ASN TYR ARG GLY ASN VAL ALA VAL
            260                 265                 270
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